APPENDIX 6

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Invariant Chain Induces B Cell Maturation by Activating a $TAF_{II}105$-NF-κB-dependent Transcription Program*

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Early stages of B cell development occur in the bone marrow, resulting in formation of immature B cells. From there these immature cells migrate to the spleen where they differentiate to mature cells. This final maturation step is crucial for the B cells to become responsive to antigens and to participate in the immune response. Recently, invariant chain (Ii), a major histocompatibility complex class II chaperone, as well as the transcription factors c-Rel and p65/RelA, were found to play a role in the final antigen-independent differentiation stage of B cells in the spleen. In this study, we investigated a possible link between Ii-dependent B cell maturation and the NF-κB pathway. Our studies indicate that Ii-induced B cell maturation involves activation of transcription mediated by the NF-κB p65/RelA homodimer and requires the B cell-enriched coactivator TBP-associated factor $_{II}105$.

In the bone marrow, B cell development can be divided into different stages, based on the rearrangement status of the IgH and IgL chain loci (1, 2) and the expression of intracellular and surface-bound markers. The first cells expressing IgM during this developmental process are the immature B cells. At this stage in their development the B cells translocate from the bone marrow to the spleen (3, 4). In the spleen, B cells are still immature and are distinguishable from their mature counterparts (3, 5–7). The subsequent differentiation event leads to mature B cells and is characterized by a series of changes in surface marker expression and in the activities of these cells. Only 5 to 10% of the newly generated immature B cells are selected into the pool of long-lived antigen-responsive mature B cells (7, 8). The molecular mechanisms controlling the selection of immature B cells and especially their differentiation in the spleen are largely unknown.

The process of B cell development is controlled by a set of transcription factors, as well as stage- and lineage-restricted genes. Recently several studies implicated NF-κB as a major regulator of the final stage of B cell maturation. Cells deficient in the NF-κB family members, c-Rel and RelA, were found to be arrested at the immature stage (9), indicating that these factors are essential for antigen-independent B cell development in the spleen. In addition, IκBα overexpression in B cells inhibited the formation of a mature B cell population (10), supporting the notion that NF-κB has an essential role in late B cell differentiation.

We have shown that invariant chain (Ii),[1] a major histocompatibility complex class II chaperone, plays a crucial role in the differentiation of immature B cells in the spleen (11). In mice lacking Ii, the development from immature to mature B cells is impaired, and B cells are arrested at an immature stage characterized by low expression levels of IgD and CD23 and poor response to T-independent antigens (11–13). Recently we succeeded in disengaging the chaperonin activity of Ii from its role in B cell maturation and demonstrated that Ii N-terminal domain is directly involved in the maturation of B cells.[2] However, at present, the molecular mechanism by which Ii induces B cell maturation is still unknown.

Because B cells that are deficient in either Ii or NF-κB family members, c-Rel and RelA, are both arrested at the immature stage in the spleen, in this study, we investigated a possible link between Ii-induced maturation and NF-κB. Indeed, our results indicate that Ii induces maturation by activating a pathway leading to up-regulation of transcription mediated by the p65 component of NF-κB and its coactivator, $TAF_{II}105$.

MATERIALS AND METHODS

Cells

Spleen cells were obtained from the various mice at 6–8 weeks of age as described previously (15). B cell population was enriched by treating the splenocyte suspension with antibodies against T cell surface molecules (anti-Thy 1.2, CD4, and CD8; Southern Biotechnology Associates, Inc.) for 1 h followed by incubation with low Tox-M complement (Cederlane) for 1 h at 37 °C.

Cell Transfection

Primary B Cells—The method used was adapted from a procedure described previously (16). Purified $Ii^{-/-}$ B cells were incubated with 50 μg/ml lipopolysaccharide from *Salmonella typhosa* (Sigma). After 48 h, the cells were washed with RPMI medium and transfected with TransFast transfection reagent (Promega) using 12 μl/4 μg of DNA (2 μg of pBabe-puro Ii construct + 2 μg of empty vector or 4 μg of empty vector) according to the manufacturer's directions. The cells were collected after 48 h and analyzed for their cell surface marker expression by FACS analysis. IκB, p65, and p50 constructs were described previously (21, 22). The percentage increase of IgD^+ cells was calculated by subtracting the *X* mean value of IgD^+ staining of empty expression plasmid

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[1] The abbreviations used are: Ii, invariant chain; FACS, fluorescence-activated cell sorter; TAF, TBP-associated factor.

[2] D. Matza, D. Lantner, Y. Bogoch, L. Flaishon, R. Heshkoviz, and I. Shachar, submitted for publication.

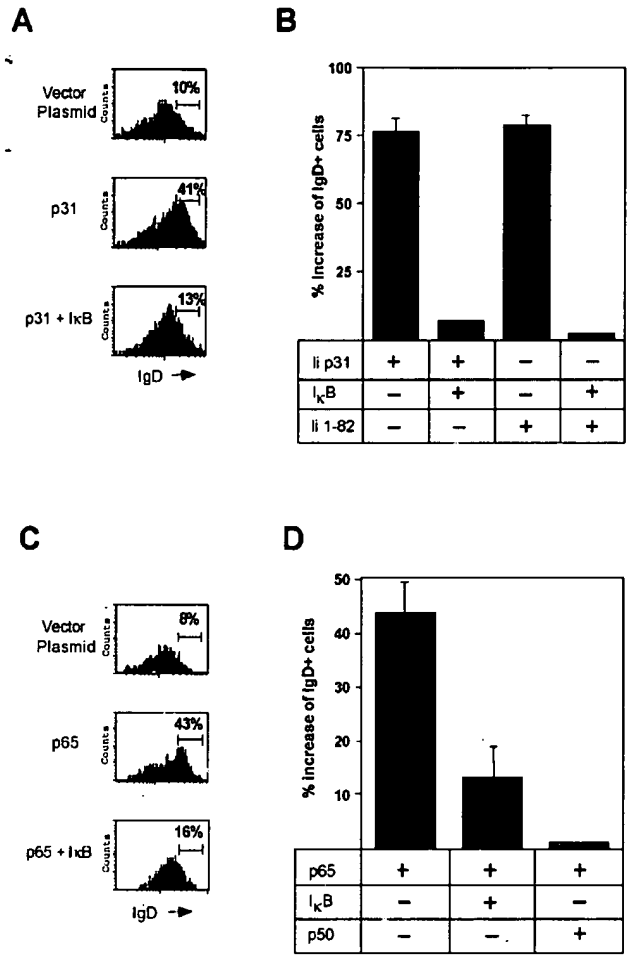

FIG. 1. **p65 homodimers are required for the B cell maturation pathway.** B cells from Ii$^{-/-}$ animals were purified and cultured. 48 h later the cells were transfected with empty expression plasmid (*Vector plasmid*), Ii p31 or Ii1–82 expression vectors alone, or cotransfected with an undegradable mutant of IκB (*A* and *B*) and empty plasmid vector, p65 alone, or p65 cotransfected with either the IκB or p50 (*C* and *D*). After 48 h cells were analyzed by FACS for IgD expression. *A* and *C* show a representative result. *B* and *D* represent five different independent transfection experiments.

and dividing it to the same *X* mean value multiplied by 100%.

293 Cells—293 cells (human embryonic kidney fibroblasts) were maintained in F12 Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfections were performed using the standard $CaPO_4$ method. For reporter assay the cells were seeded in a 24-well multidish, using a total of 1 μg of plasmid. Usually, 25 ng of NF-κB or AP-1 reporters were used, together with 500 or 800 ng of Ii expression plasmid, and the total amount of DNA was kept by adding pBabe vector. When Gal4 luciferase reporter was analyzed 5 ng of the reporter and 1–5 ng of DBD fusion plasmids were used (21, 22).

Immunofluorescence and Flow Cytometry

Invariant chain-deficient transfected cells were stained with the 9.1 anti-IgD (Pharmingen) antibody as was described previously (17).

Electrophoresis Mobility Shift Assay

Nuclear extracts were prepared as described (18) from Ii$^{-/-}$ primary B cells or from 293 cells. A double stranded oligonucleotide, containing the NF-κB site, was labeled with Klenow fragment of DNA polymerase I as a probe. The sequence of the oligonucleotide is as follows: 5′ GATCCAGAGGGGACTTTTCCGAGAG 3′; 5′ GATCCTCTCGGAAAGTCCCCTCTG 3′.

RESULTS AND DISCUSSION

Spleen B cells from Ii-deficient mice are arrested at the immature stage. We have developed an *in vitro* system to analyze B cell maturation. In this assay primary immature B cells from Ii-deficient mice can differentiate to mature cells in culture, by introducing Ii expression plasmid (Fig. 1, *A* and *B*).[2] To investigate the involvement of NF-κB in Ii-induced B cell maturation, we cotransfected an undegradable mutant form of IκB, an inhibitor of NF-κB, along with p31 Ii. In addition we introduced the 1–82 Ii fragment (Ii1–82) lacking its lumenal domain. This IκB mutant abolished both p31- and Ii1–82-mediated maturation (Fig. 1*B*). As p65/RelA is a direct target for inhibition by IκB and is also associated with the process of B cell maturation, it is plausible that NF-κB p65/RelA is a downstream effector of Ii-induced maturation. If so overexpression of p65/RelA should induce maturation of Ii$^{-/-}$ primary immature B cells. Indeed, p65/RelA overcame the developmental arrest of the Ii$^{-/-}$ B cells and directly induced maturation, and its effect was abolished by dominant IκB (Fig. 1*C*). To characterize the specificity of NF-κB dimers in inducing maturation of Ii-deficient primary B cells, we analyzed maturation induction by p50·p65 dimer. In contrast to the p65·p65 complex, the heterodimeric complex, p65·p50, was unable to induce differentiation of B cells (Fig. 1*D*).

To address the mechanism by which Ii induces B cell maturation via NF-κB, we examined the effect of Ii expression on NF-κB transcription activity. For these studies we used human 293 cells that do not express the endogenous Ii gene. The use of non-B cells for studies of B cell-specific transcription has been shown in many studies to provide valuable functional information for transcription factors and transcription modulators (19). A luciferase reporter plasmid containing two tandem NF-κB sites and a minimal core promoter was transfected, along with the expression plasmid for Ii, and luciferase activity was measured 24 h later. As a control NF-κB-mutated or AP-1-dependent reporter plasmids were similarly analyzed. Microscopic examination showed Ii-transfected cells to have the same healthy morphology as untransfected cells. As shown in Fig. 2*A* Ii enhanced the constitutive NF-κB-dependent activity in a dose-dependent manner (columns 1–3). The NF-κB-mutated reporter displayed negligible activity and was not affected by Ii expression (columns 4–6). The AP-1-dependent reporter gene was not affected by Ii expression (columns 7–9) confirming the specificity of the effect of Ii on NF-κB. As the N-terminal segment of Ii (p31 1–82) is capable of promoting B cell maturation similar to the wild type protein, we next analyzed the effect of this mutant on NF-κB transcription activity. As can be seen in Fig. 2*B*, amino acids 1–82 enhanced the constitutive NF-κB-dependent activity to the same extent as the full-length protein. Moreover, a construct of Ii lacking part of its cytosolic domain, amino acids 2–17 (Δ 2–17), was unable to enhance this activity (Fig. 2*B*). Thus, Ii N-terminal 17 amino acids are important for induction of NF-κB transcription activity.

A major pathway for NF-κB activation involves induction of its nuclear localization. To determine whether Ii expression affects this process, nuclear extracts were prepared from 293 cells transfected with either empty or invariant chain expression plasmids (efficiency of transfection was above 80%). These extracts were used in electrophoresis mobility shift assay with an NF-κB-specific probe. Similar levels of nuclear NF-κB DNA binding activity were found in control or Ii-expressing cells, indicating that Ii does not induce nuclear transport of NF-κB (Fig. 2*C*). The observed DNA binding activity is NF-κB-specific as incubation of these extracts with antibodies against the p65/RelA component of NF-κB resulted in a supershifted complex (data not shown). Thus, Ii stimulates transcription activity of NF-κB through a pathway that is independent of NF-κB nuclear transport.

To investigate the mechanism by which Ii controls NF-κB,

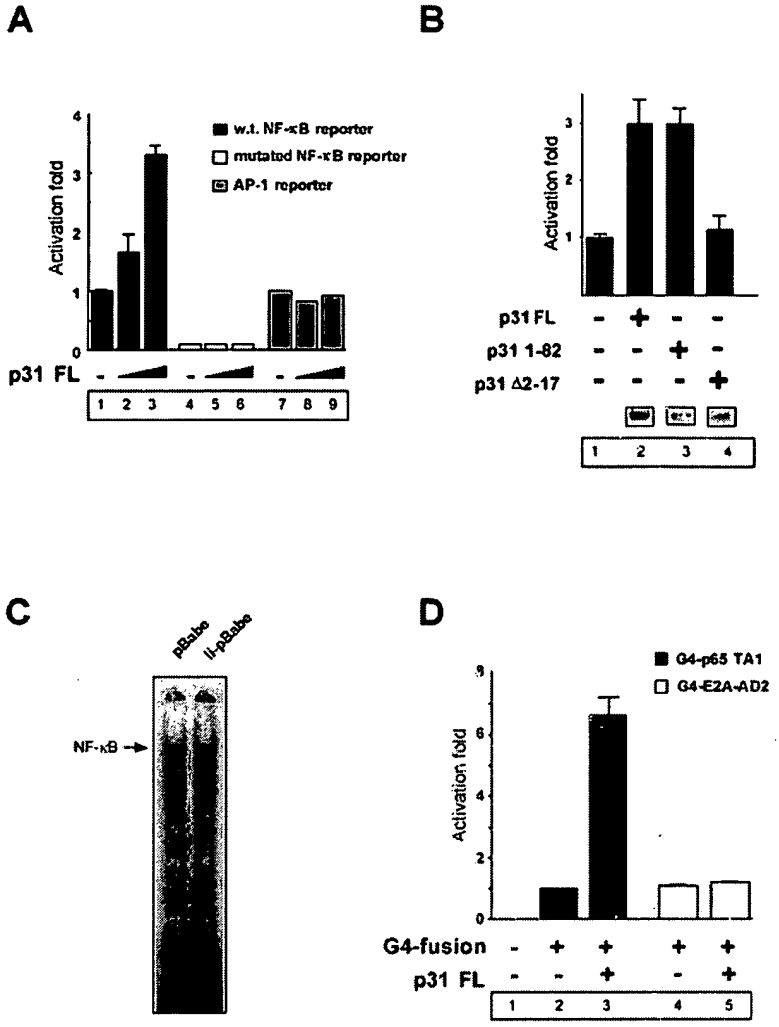

FIG. 2. **Stimulation of NF-κB-dependent transcription by Ii.** *A*, wild type (*w.t.*) and mutated NF-κB reporter plasmids containing two NF-κB-binding sites and AP-1 reporter plasmid were transfected into human 293 cells, together with either empty expression vector or with increasing amounts of Ii expression vector (0.5 and 1 μg). Luciferase activity was measured, and the activity of the reporter alone was normalized to 1. *B*, wild type NF-κB reporter plasmids containing two NF-κB-binding sites were transfected into human 293 cells, together with either empty expression vector or with Ii p31 or Ii1–82 amino acids or Ii Δ 2–17 expression vector (1 μg). The expression of different Ii-derived proteins was confirmed by Western blot analysis (*lower panel*) using IN1 antibody for full-length (FL) and 1-82 and anti-CD74 (Santa Cruz Biotechnology) for Δ2-17. *C*, 293 cells were transfected with either empty vector or Ii expression plasmid, and nuclear extracts were prepared. Transfection efficiency was above 80% as determined by cotransfected green fluorescent protein plasmid. The extracts were subjected to electrophoresis mobility shift assay using an NF-κB-specific probe. *D*, 293 cells were transfected with the luciferease reporter plasmid containing five Gal4 binding sites, together with unsaturated amounts of G4-p65 TA1 (the TA1 activation domain of p65/RelA fused to the GAL4 G4-E2A-AD2 DNA binding domain or in the presence or absence of Ii expression plasmid (1 μg). Luciferase units were measured, and transcriptional activity of G4-p65 TA1 and G4-E2A-AD2 were normalized to 1. The results shown represent the average of at least four independent experiments with similar results.

we examined whether the effect of Ii is directed to the transcriptional activation function of p65/RelA. For this purpose we used an expression plasmid that encodes a fusion of the C-terminal transactivation domain of p65/RelA (TA1) with the DNA-binding domain of the yeast transcription factor Gal4. As a control, the E2A activation domain fused to Gal4 was used. These plasmids were transfected into 293 cells, along with a luciferase reporter containing upstream Gal4-binding sites in the presence or absence of Ii plasmid. As shown in Fig. 2*D*, Ii strongly and specifically stimulated the activity of the Gal4p65 but not Gal4E2A fusion proteins. These results indicate that the effect of Ii on NF-κB activity is at least in part targeted to the activation function of p65/RelA.

TAF$_{II}$105 is a subunit of the general transcription factor TFIID, which is enriched in B cells (20). TAF$_{II}$105 appears to be present only in a subset of TFIID complexes and therefore is likely to function in the context of specific genes. TAF$_{II}$105 was shown to act as an activation domain-specific coactivator of p65/RelA homodimers. This function of TAF$_{II}$105 involves its direct interaction with the p65/RelA activation domain (21, 22). To examine whether TAF$_{II}$105 is also involved in B cell differentiation, we employed two dominant negative forms of TAF$_{II}$ 105 lacking the TFIID interaction region (TAF$_{II}$105ΔC) in the Ii-mediated B cell maturation assay. TAF$_{II}$105ΔCΔ 452–472 is a double mutant of TAF$_{II}$105ΔC that is unable to bind p65/RelA (TAF$_{II}$105ΔC-ΔNF-κB; see Ref. 22). Cotransfection of TAF$_{II}$105ΔC with the p31 or 1–82 Ii fragment abolished maturation (Fig. 3*A*). Apparently, suppression of the endogenous TAF$_{II}$105 activity by the competing dominant negative TAF$_{II}$105ΔC prevented the maturation process. By contrast, cotransfection of TAF$_{II}$105ΔC-ΔNF-κB with p31 Ii did not interfere with the maturation process (Fig. 3*A*). This result further supports the involvement of p65/RelA and the NF-κB pathway in Ii-induced B cell maturation.

Next, we analyzed the effect of TAF$_{II}$105ΔC on Ii-induced NF-κB transcriptional activity using a luciferase reporter assay. As shown in Fig. 3*B* Ii-enhanced NF-κB-dependent activity was dramatically inhibited when cotransfected with TAF$_{II}$ 105ΔC. In contrast, TAF$_{II}$105ΔC impaired in NF-κB binding (TAF$_{II}$105ΔC-ΔNF-κB) did not interfere with the Ii-induced activity. Together, these results strongly suggest that NF-κB and TAF$_{II}$105 are directly involved in Ii-mediated B cell maturation.

The role of Ii as a chaperone for major histocompatibility complex class II molecules has been well characterized. Recently, we have described a novel role for Ii. In mice lacking this chain, B cell maturation is impaired, and their secondary lymphoid organs are enriched with immature B cells (11). The data presented in this study reveal that Ii is most likely a signaling molecule that regulates this maturation step by inducing a specific gene expression program. Essential elements in this process are the p65 component of NF-κB and its coactivator TAF$_{II}$105, a subunit of the basal transcription factor TFIID, which is highly expressed in B cells. Several lines of

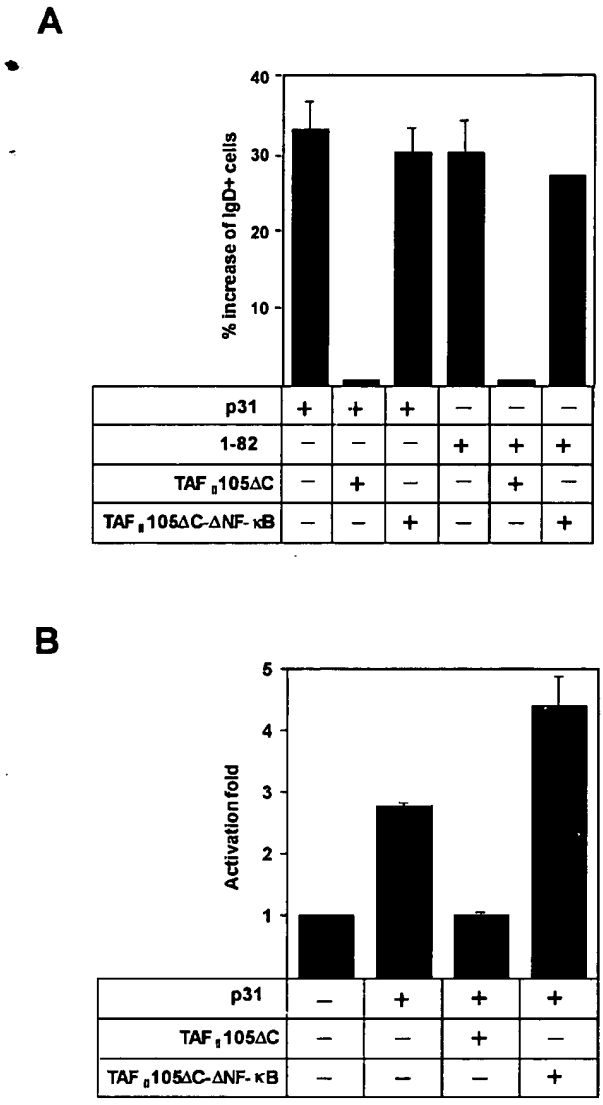

FIG. 3. **Ii stimulates B cell maturation through p65/RelA and TAF$_{II}$105.** Transfections were performed as described for Fig. 1. Cells were transfected with (*A*) empty expression plasmid, p31 or Ii1–82 alone, or together with the dominant negative mutant of TAF$_{II}$105 (TAF$_{II}$105ΔC) or TAF$_{II}$105ΔC Δ 452–472 (TAF$_{II}$105ΔC-ΔNF-κB). The cells were FACS analyzed as described for Fig. 1 for IgD expression. The *graph* represents the results of four independent transfection experiments. *B*, wild type NF-κB reporter plasmid containing two NF-κB-binding sites was transfected into human 293 cells, together with either empty expression vector or with Ii expression vector (1 μg) with or without TAF$_{II}$105ΔC (1 μg) or TAF$_{II}$105ΔC-ΔNF-κB (1 μmg). The *graph* represents the results of three independent transfection experiments.

evidence support this conclusion. First, maturation by Ii was suppressed by NF-κB-specific inhibitors, IκB and the dominant negative mutant of TAF$_{II}$105 (TAF$_{II}$105ΔC). However, mutant TAF$_{II}$105ΔC lacking the p65 interaction domain failed to inhibit Ii-induced maturation. Furthermore, we show that p65/RelA could efficiently overcome the B cell maturation block of Ii-deficient B cells. The differentiation induced by p65 is similarly inhibited by IκB and by TAF$_{II}$105ΔC (data not shown). Interestingly, only p65·p65 but not p65·p50 NF-κB dimeric complexes have the capacity to activate the maturation process, a result consistent with the finding that TAF$_{II}$105 preferentially associates with p65/RelA homodimer (21). Moreover, these findings indicate that the activity of p65·p65 is not redundant with that of the p50·p65 complex in the regulation of this stage of B cell differentiation. In conclusion, a transcription complex of p65·p65 and TAF$_{II}$105 is an essential downstream component of a signaling pathway stimulated by Ii that leads to specific gene activation.

NF-κB is a family of transcription factors that is activated by a broad range of extracellular signals. A major pathway in regulating NF-κB activity involves its nuclear transport (23). Several studies have characterized additional pathways for inducing the activation potential of the p65 subunit of NF-κB. The activation domain of this molecule was shown to be affected by several signaling pathways including cAMP-dependent protein kinase (24), protein kinase C (25), Ras (25–27), and the mitogen-activated protein kinases p38 and Erk (14). These signaling pathways enhance the activation function of p65. Our experiments show that one possible pathway utilized by Ii involves modulation of the p65/RelA activation domain. We speculate that this event augments the interaction of p65 with coactivator molecules such as TAF$_{II}$105, resulting in activation of specific genes required for B cell maturation. We suggest that Ii initiates a signaling cascade that is transmitted to the nucleus and activates protein kinases leading to modulation of either the p65 activation domain or its coactivators. The role of p65/RelA in the transition from immature to mature B cells in the spleen is consistent with recent studies showing that c-Rel and RelA play a crucial role in the final stages of B cell development (9). In that study c-Rel and RelA double deficient B cells, like Ii-deficient cells (11–13) were arrested at the immature stage. The nature of the Ii signal essential for the maturation, as well as the pathway by which the Ii signal is transmitted to the nucleus, is still open to questions.

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